

Association of extracellular-superoxide dismutase phenotype with the endothelial constitutive nitric oxide synthase polymorphism

Tetsuo Adachi^{a,*}, Xing Li Wang^b

^aLaboratory of Clinical Pharmaceutics, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu, Japan

^bDepartment of Cardiovascular Medicine, University of New South Wales, Prince Henry/Prince of Wales Hospital, Sydney, Australia

Received 29 June 1998; revised version received 13 July 1998

Abstract The distribution of extracellular-superoxide dismutase (EC-SOD) levels in healthy Australian subjects was consistent with two distinct phenotypes in which the smaller group of subjects (3.3%) had 15-fold higher levels. The EC-SOD levels in individuals homozygous for endothelial constitutive nitric oxide synthase 4a (ecNOS4a), a rare allele for ecNOS repeat polymorphism at intron 4, were significantly lower than those in ecNOS4A/a and ecNOS4A/A subjects. Furthermore, NO levels were negatively correlated with the EC-SOD levels in common EC-SOD phenotype subjects. Whilst the mechanism remains speculative, it is possible that there is a significant interaction between EC-SOD and ecNOS, or that common factor(s), either genetic or environmental, influence both of them.

© 1998 Federation of European Biochemical Societies.

Key words: Extracellular-superoxide dismutase; Endothelial constitutive nitric oxide synthase; Polymorphism; Peroxynitrite; Coronary artery disease

1. Introduction

The occurrence of highly reactive oxygen species and their destruction by anti-oxidants is in equilibrium in healthy mammalian organisms. Disturbance of this homeostasis can cause numerous disorders. It has been suggested that free radicals are involved in various cardiovascular diseases including atherosclerosis. Thus, the defense systems against active oxygen species are critical for protecting blood vessel walls against oxidative damage.

Nitric oxide (NO) is produced by a variety of cells in the vascular system, including endothelial cells, macrophages, smooth muscle cells, platelets and fibroblasts. Three NO synthases (NOSs), neuronal constitutive NOS (ncNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial constitutive NOS (ecNOS or NOS3), are responsible for NO biosynthesis in these cells [1]. NO is produced constitutively and contributes to the modulation of vasomotor tone and to inhibition of platelet and leukocyte aggregation and adhesion to endothelium, properties that have been shown to be anti-atherogenic [2]. While iNOS is not normally expressed, it can be induced by cytokines and lipopolysaccharides in some pathological processes, such as circulatory shock and inflammation [3].

NO reacts extremely rapidly with superoxide to produce peroxynitrite, a potential mediator of oxidant-induced cellular injury, at or near the diffusion-limited rate ($4.3\text{--}6.7 \times 10^9 \text{ M}^{-1}$

s^{-1}). Peroxynitrite is a potent oxidant, capable of oxidizing thiols [4,5] and DNA bases [6], and of causing tyrosine nitration [7] and initiating lipid peroxidation [8]. Superoxide dismutase (SOD) is a potent scavenger of superoxide at the rate of $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. NO may compete with SOD for superoxide, thereby removing superoxide and thus supporting its antioxidant role [9]. Extracellular-SOD (EC-SOD or SOD3) is the principal enzymatic scavenger of superoxide in the extracellular space [10]. In the vasculature, EC-SOD is in equilibrium between the plasma phase and heparan sulfate proteoglycans in the glycocalyx of the endothelium [11]. Molecular genetic studies of EC-SOD have shown that a single base substitution causing substitution of glycine for arginine-213 (R213G) in the heparin-binding domain of this enzyme causes the extremely high plasma level of EC-SOD [12–15]. The high plasma EC-SOD phenotype was found in a small proportion of healthy subjects, 6% in Japanese [12], and 2% in Swedish [14]. It is unclear whether this phenotype exerts beneficial or deleterious effects on individuals.

The endothelium is a primary target of vascular injury. Elevation of NO and/or depression of SOD in endothelium or its microenvironment might cause the pathogenesis of vascular disorders. The hypothesis of interactions between NO and SOD is supported by the finding that expressions of EC-SOD and iNOS were elevated simultaneously in human and rabbit atherosclerotic lesions [16]. We have reported a genetic contribution of ecNOS to plasma NO levels, as indicated by levels of plasma nitrite and nitrate (NOx), and an association of the rare allele of a 27-bp repeat in intron 4 with a tobacco smoking-dependent increased risk of coronary artery diseases (CAD) [17,18]. The balance of these two systems would clearly be important in many pathological processes such as atherogenesis. In the present study, we explored associations between ecNOS polymorphism and EC-SOD levels in an Australian Caucasian population.

2. Materials and methods

2.1. Nuclear families in the study

We collected blood samples from 242 members of 57 nuclear families. These families were healthy volunteers recruited from our Heart Health Education Program for family-based primary coronary prevention. They were selected for the current study on the basis that all were of white European origin and residing in Sydney, and none were current smokers. They were advised to remain on their usual diet before the blood collection and all were healthy at the time of study. Written consent was obtained from every child's parent for this study. The study was approved by the Ethics Committee of the University of New South Wales. A 4-ml venous blood sample was drawn into an EDTA-containing sample tube after an overnight fast (12–14 h). The blood sample was centrifuged within 2 h and plasma was stored at -70°C in aliquots until analysis. DNA was extracted from the frozen cellular blood component by a salting-out method [17]. The extracted DNA was stored at 4°C until analysis.

*Corresponding author. Fax: (81) (58) 237-5979.
E-mail: adachi@gifu-pu.ac.jp

Abbreviations: EC-SOD, extracellular-superoxide dismutase; NO, nitric oxide; NOS, nitric oxide synthase; CAD, coronary artery diseases; NOx, nitrite and nitrate

2.2. Assay of EC-SOD

The EC-SOD concentration was determined by the enzyme-linked immunosorbent assay (ELISA) as described previously [19].

2.3. Genotyping of the 27-bp polymorphism in intron 4 of the *ecNOS* gene

A polymerase chain reaction (PCR) method was used for the genotyping of the repeat polymorphism as described previously [18]. We used oligonucleotide primers that flank the region of the 27-bp repeat in intron 4 of the *ecNOS* gene. The PCR products were electrophoresed on 8% polyacrylamide gels and visualized by silver staining. There are two alleles differing by one repeat, i.e. differing by 27 bp in size. We have denoted these two alleles as *ecNOS4a* for the rare four repeat allele and *ecNOS4A* is the common allele.

2.4. Determination of plasma NO_x levels

Since NO is unstable and quickly oxidized to nitrate and nitrite after production, to estimate plasma NO levels, circulating NO_x was determined using a modification of the method described by Wang et al. [20]. Nitrate was measured as nitrite after enzymatic conversion by nitrate reductase, and nitrite was measured after deproteinization using the Griess color reaction, which was read at a wavelength of 540 nm. Values obtained by this procedure represent the sum of nitrite and nitrate derived from NO.

2.5. Statistical analyses

The levels of SODs and NO_x measured are presented as means ± S.D., and differences among subgroups were compared by the Kruskal-Wallis test for three subgroups (*ecNOS4A/A*, *ecNOS4A/a* and *ecNOS4a/a*), or by Student's *t*-test for two groups (low EC-SOD and high EC-SOD). A general factorial design of ANOVA and a linear regression model were used to assess and control for the confounding factors.

3. Results

Two hundred and forty-two individuals were included in the current study. Serum EC-SOD levels ranged from 29.9 to 1798 µg/l and were distributed in two discrete groups of low-level (29.9–152.1 µg/l, *n* = 234) and high-level (940.2–1798

µg/l, *n* = 8, frequency 3.3% of tested samples). All of the tested high-level EC-SOD individuals were found to carry the R213G mutation, whereas none of the low-level EC-SOD subjects had this mutation.

The frequency distribution of the *ecNOS4* genotypes was 0.690 (*n* = 167) for *ecNOS4A/A*, 0.285 (*n* = 69) for *ecNOS4A/a* and 0.025 (*n* = 6) for *ecNOS4a/a*. The *ecNOS4a/a* homozygotes exhibited two-fold higher levels of the circulating plasma NO_x than the other subjects (Table 1). Age and sex distributions of the two groups of EC-SOD level and the three groups of *ecNOS4* genotype were not significantly different.

Table 1 shows that EC-SOD levels of *ecNOS4a/a* subjects were significantly lower than those of the *ecNOS4A/A* and *ecNOS4A/a* groups. When subgroups of common (low level) and mutant (high level) EC-SOD phenotypes were considered separately, there were no *ecNOS4a/a* individuals with the mutant EC-SOD phenotype. To confine the comparison to individuals of the common phenotype, EC-SOD levels in *ecNOS4a/a* subjects were still significantly lower than those in *ecNOS4A/A* subjects and tended to be lower than those in *ecNOS4A/a* subjects (Table 1). Furthermore, levels of NO_x were negatively and significantly correlated with the levels of EC-SOD ($\beta = -0.196$, $r = 0.181$, $F = 7.799$, $P = 0.006$) in the subgroup of common phenotype individuals. In a general factorial design of ANOVA, the *ecNOS4* polymorphism remained a significant contributor to EC-SOD levels ($F = 4.57$, $P = 0.011$) after controlling for age ($F = 77.84$, $P = 0.0001$) and sex ($F = 5.21$, $P = 0.023$). The adjusted means ± S.E.M. of EC-SOD for *ecNOS4A/A*, *A/a* and *a/a* were 80.9 ± 1.7 µg/l, 78.9 ± 2.6 µg/l and 54.1 ± 8.8 µg/l respectively.

Whilst none of the subjects in the high EC-SOD group were *ecNOS4a/a* homozygotes, the frequency of the homozygotes in the common subgroup was 2.56%. The plasma NO_x levels were not different in the two groups (Table 2).

Table 1
Relation between the *ecNOS* polymorphism and EC-SOD level

	<i>ecNOS4A/A</i>	<i>ecNOS4A/a</i>	<i>ecNOS4a/a</i>	<i>P</i> -value
Total				
Sample number	167	69	6	
Age	24.7 ± 16.2	25.9 ± 15.7	23.2 ± 14.2	0.7360
Male/female	91/76	38/32	5/1	
NO _x (µmol/l)	33.7 ± 17.0	36.6 ± 28.4	74.9 ± 56.3	0.0834
EC-SOD (µg/l)	135.4 ± 264.1	91.1 ± 106.6	57.8 ± 20.7 ^{b,c}	0.0202
Common EC-SOD (µg/l) ^a	81.4 ± 25.2	78.6 ± 24.6	57.8 ± 20.7 ^b	0.0317
Number of subjects without rare EC-SOD allele	160	68	6	
Number of subjects with rare EC-SOD allele	7	1	0	
Males				
Sample number	91	38	5	
EC-SOD (µg/l)	147.9 ± 277.2	86.6 ± 26.2 ^b	59.1 ± 22.9 ^b	0.0688
Common EC-SOD (µg/l) ^a	82.2 ± 25.8	86.6 ± 26.2	59.1 ± 22.9	0.0611
Number of subjects without rare EC-SOD allele	86	38	5	
Number of subjects with rare EC-SOD allele	5	0	0	
Females				
Sample number	76	32	1	
EC-SOD (µg/l)	120.5 ± 248.5	96.5 ± 157.6	51.1	0.0446
Common EC-SOD (µg/l) ^a	80.5 ± 24.5	68.4 ± 18.2 ^b	51.1	0.0336
Number of subjects without rare EC-SOD allele	74	31	1	
Number of subjects without common EC-SOD allele	2	1	0	

^aEC-SOD levels in the subgroup without rare EC-SOD allele and their plasma EC-SOD level distributed below 150 µg/l.

^b $P < 0.05$ vs. *ecNOS4A/A* group.

^c $P < 0.05$ vs. *ecNOS4A/a* group by Student's *t*-test.

Table 2
Relation between the EC-SOD phenotypes, ecNOS polymorphism, and other factors

	Low EC-SOD	High EC-SOD	P-value
Sample number	<i>n</i> = 234	<i>n</i> = 8	
Age	25.2 ± 16.0	19.9 ± 16.2	0.3917
Male/female	129/105	5/3	
EC-SOD (μg/l)	80.0 ± 25.1	1317 ± 262.9	< 0.0001
NOx (μmol/l)	35.7 ± 23.4	33.6 ± 16.0	0.7392
Frequency of ecNOS4a/a (%)	2.56%	0%	

4. Discussion

We reported previously that the frequency of ecNOS4a/a homozygotes in the CAD population was six times higher than that in the healthy population [17], which suggested the contribution of ecNOS to the development of CAD. We further indicated the significant genetic contribution of ecNOS to plasma NOx levels by showing that NOx levels in ecNOS4a/a individuals were twice as high as levels in individuals with the ecNOS4A allele [18]. Despite many environmental factors that alter the plasma NO level, approximately 30% of the variance in NO level is due to the ecNOS4 polymorphism [18]. The present study identified a significant association between ecNOS polymorphism and EC-SOD phenotype. Whilst the mechanism remains speculative, it is possible that these two factors interact *in vivo*, or that common factor(s), either genetic or environmental, may influence both ecNOS and EC-SOD.

Serum EC-SOD levels in subjects carrying the R213G mutation were about 15-fold higher than in subjects without the mutation. However, the mutant EC-SOD has the same enzymatic specific activity as the common phenotype enzyme [14]. The present results showed a significantly inverse correlation between circulating NOx levels and EC-SOD levels in the subgroup of common (low) EC-SOD phenotype, although the EC-SOD mutation did not influence the plasma NOx levels (Table 2). Whilst continuous release of NO by the endothelium into the circulation has important biological functions relevant to the prevention of atherogenesis, high circulating NO levels are toxic. This toxic effect is thought to be at least partly mediated by active end metabolites of NO, which may initiate lipid peroxidation in low-density lipoprotein [21,22]. It can be speculated that these oxidative metabolites may always be produced and toxic even when the NO levels are within physiological range, and this effect may be attenuated by constant EC-SOD production. It is possible that a high level of EC-SOD in plasma quickly dismutates the superoxide produced by stimulated neutrophils and prevents the production of toxic peroxynitrite in the circulation. Above findings are consistent with the fact that the ecNOS4a homozygotes, whose plasma NOx level is two-fold higher and EC-SOD level is significantly lower than those of individuals with the ecNOS4A allele, are predisposed to coronary injury. Additional oxidant stress such as cigarette smoking will raise the risk further.

In summary, our study demonstrated a highly significant association between plasma EC-SOD and the ecNOS4 polymorphism, in which low EC-SOD levels were related to the rare ecNOS4a allele, which was also associated with high NOx levels. We further showed a significant negative correlation between the plasma NOx and EC-SOD levels in subjects with common (low-level) EC-SOD phenotypes. Whilst further

studies are needed to explore the mechanisms, these findings suggest that NO and EC-SOD may function conjointly in physiology and pathology.

Acknowledgements: The project is supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (T.A.) and by a grant from National Health and Medical Research Council Australia, and a Cardiovascular Research Fund from Eastern Heart Clinic, Prince of Wales Hospital, Sydney, Australia (X.L.W.).

References

- [1] Kerwin Jr., J.F., Lancaster Jr., J.R. and Feldman, P.L. (1995) *J. Med. Chem.* 38, 4343–4362.
- [2] Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) *Pharmacol. Rev.* 43, 109–142.
- [3] Nathan, C. and Xie, Q.-W. (1994) *J. Biol. Chem.* 269, 13725–13728.
- [4] Whiteman, M. and Halliwell, B. (1997) *FEBS Lett.* 414, 497–500.
- [5] Gatti, R.M., Radi, R. and Augusto, O. (1994) *FEBS Lett.* 348, 287–290.
- [6] King, P.A., Jamison, E. and Strahs, D. (1993) *Nucleic Acids Res.* 21, 2473–2478.
- [7] Halliwell, B. (1997) *FEBS Lett.* 411, 157–160.
- [8] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) *Arch. Biochem. Biophys.* 288, 481–487.
- [9] Wink, D.A., Hanbauer, I., Krishna, M.C., DeGraff, W., Gamson, J. and Mitchell, J.B. (1993) *Proc. Natl. Acad. Sci. USA* 90, 13–17.
- [10] Strålin, P., Karlsson, K., Johansson, B.O. and Marklund, S.L. (1995) *Arterioscler. Thromb. Vasc. Biol.* 15, 2032–2036.
- [11] Karlsson, K. and Marklund, S.L. (1987) *Biochem. J.* 242, 55–59.
- [12] Adachi, T., Ohta, H., Yamada, H., Futenma, A., Kato, K. and Hirano, K. (1992) *Clin. Chim. Acta* 212, 89–102.
- [13] Yamada, H., Yamada, Y., Adachi, T., Goto, H., Ogasawara, N., Futenma, A., Kitano, M., Hirano, K. and Kato, K. (1994) *Jpn. J. Hum. Genet.* 40, 177–184.
- [14] Sandström, J., Nilsson, P., Karlsson, K. and Marklund, S.L. (1994) *J. Biol. Chem.* 269, 19163–19166.
- [15] Folz, R.J., Peno-Green, L. and Crapo, J.D. (1994) *Hum. Mol. Genet.* 3, 2251–2254.
- [16] Luoma, J.S., Strålin, P., Marklund, S.L., Hiltunen, T.P., Särkioja, T. and Ylä-Herttuala, S. (1998) *Arterioscler. Thromb. Vasc. Biol.* 18, 157–167.
- [17] Wang, X.L., Sim, A.S., Badenhop, R.F., McCredie, R.M. and Wilcken, D.E.L. (1996) *Nature Med.* 2, 41–45.
- [18] Wang, X.L., Mahaney, M.C., Sim, A.S., Wang, J., Wang, J., Blangero, J., Almasy, L., Badenhop, R.B. and Wilcken, D.E.L. (1997) *Arterioscler. Thromb. Vasc. Biol.* 17, 3147–3153.
- [19] Adachi, T., Ohta, H., Yamada, H., Futenma, A., Kato, K. and Hirano, K. (1992) *Clin. Chim. Acta* 212, 89–102.
- [20] Wang, J., Brown, M.A., Tam, S.H., Chan, M.C. and Whitworth, J.A. (1997) *Clin. Exp. Pharmacol. Physiol.* 24, 418–420.
- [21] White, C.R., Brock, T.A., Chang, L.-Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W.A., Gianturco, S.H., Gore, J., Freeman, B.A. and Tarpey, M.M. (1994) *Proc. Natl. Acad. Sci. USA* 91, 1044–1048.
- [22] Darley-Usmar, V.M., Hogg, N., O’Leary, V.J., Wilson, M.T. and Moncada, S. (1992) *Free Radical Res. Commun.* 17, 9–20.